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Monoclonal Antibody Against Pertussis Toxin: Effect on Toxin Activity and Pertussis Infections

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Antibody-producing hybridomas of myeloma SP2/O and spleen cells of BALB/c mouse immunized with pertussis toxoid and pertussis toxin were selected by the binding ability of the monoclonal antibody to the subunit protein of the toxin. Two monolconal antibodies, 1B7 and 3F10, specific for a subunit which has no binding activity to haptoglobin and sheep erythrocytes, named S1, and one antibody, 1H2, for a subunit related to the binding activity of the pertussis toxin molecule to haptoglobin or sheep erythrocytes, named S4, were examined for mouse protective activity against pertussis infection. Antibody 1B7 not only neutralized leukocytosis-promoting and islet-activating activities of the toxin but also protected mice against intracerebral and aerosol challenge with *Bordetella pertussis*. The antibody, furthermore, showed therapeutic effects on mice showing severe clinical signs with pertussis infection. The other two antibodies, 3F10 and 1H2, showed neither neutralizing nor protecting activity, nor significant synergistic effects on antibody 1B7.

Pertussis vaccine composed of killed cells has been playing a role in the reduction of whooping cough for more than 40 years throughout the world. At the same time, however, it is one of the most rejected of vaccines because of its adverse reactions. It is now time that the whole-cell vaccine be replaced by a more defined vaccine that is composed of specific components and is able to have its protective potency evaluated by means of purified reference protective antigens or antibodies. In Japan, a pertussis vaccine in use since 1981 has had some adverse effects reduced by removal of the endotoxin from a fraction of culture supernatant of *Bordetella pertussis* phase I cells and inactivation of some of the toxicity with Formalin (16). The main components of the vaccine are formalinized pertussis toxin (PT) and filamentous hemagglutinin (FHA). Now we understand that PT is the most potent antigen and FHA is a helpful protective antigen and that their antibodies play an important role in protecting mice from infection and disease caused by the pathogen (12, 17).

PT has been one of the most important subjects in our laboratory, not only as an important protective antigen but also as a unique protein with diverse biological activities in the physiological regulation system (10, 19, 21). For these studies, we have attempted to obtain subunit proteins of the toxin (18) and monoclonal antibodies (MAbs) to the toxin as tools for analysis of the structure-function relationships of the toxin.

In the present study, three different MAbs to PT were employed for our study of the role of anti-PT antibody in protection of mice against pertussis. One MAb, named 1B7, not only neutralized leukocytosis-promoting (LP) and islet-activating (IA) activities of PT, but also protected mice from intracerebral (i.c.) and aerosol challenge with *B. pertussis* cells. MAb 1B7 was as potent a protective antibody as polyclonal conventional anti-PT antibody which was sufficient to prevent pertussis in mice.

MATERIALS AND METHODS

Preparation of PT and FHA. PT and FHA were purified from the culture supernatant of *B. pertussis* Tohama phase I by successive column chromatography with hydroxylapatite, haptoglobin (Hp)-Sepharose 4B, and Sepharose 4B, as reported previously (14). The purified PT gave a single band on polyacrylamide gel electrophoresis at pH 4.0. The molecular weight of the purified PT was 107,000 (1) in polyacrylamide gel electrophoresis at pH 4.5 by the procedure of Hedrick and Smith (6). The purified PT was dialyzed against 0.1 M phosphate buffer containing 0.5 M NaCl and 50% glycerin (pH 7.0). The glycerinated PT was adjusted to a protein concentration of 200 μg/ml based on the protein concentration before dialysis, and it was used as the reference PT throughout this experiment since it can be kept at −20°C without any change in toxin activity. The reference PT used in this paper was named PT Ref 3, and its LP activity and Hp binding activity in the enzyme-linked immunosorbent assay method (Hp-ELISA) were 7,000 LP U/ml and 7,000 Hp-ELISA U/ml, respectively (see below for the assay method of PT activities). For the estimation of activities of PT Ref 3, PT Ref 1 with a titer set arbitrarily at 3,000 LP U/ml and 3,000 Hp-ELISA U/ml as described previously (13) was used as reference PT.

In vitro assay of PT activities. Binding activity of PT or its subunits to Hp (13) or sheep erythrocyte membrane (SRBCM) was assayed by the ELISA method (22). PT or subunit preparations diluted with phosphate-buffered saline (PBS) containing 0.05% Tween 20 were allowed to react with microplates coated with Hp (2 μg/ml) or SRBCM (20 μg/ml), anti-PT goat immunoglobulin G (IgG), alkaline phosphatase-anti-goat IgG conjugate, and *p*-nitrophenylphosphate, as reported previously (13). By measurement of absorbance at 405 nm with an autoreader (MR 580; Dynatech Laboratories, Inc.), dose-response curves were drawn on log-log graph paper. The binding activity was estimated from the curves by the parallel line assay method, using PT Ref 3 and expressed by Hp-ELISA units or SRBCM-ELISA units, respectively.

The hemagglutination (HA) test was performed by the following procedure. Twenty five microliters of a 0.35% (vol/vol) suspension of washed erythrocytes from 1-day-old chickens was added to 50 μl of the toxin diluted serially with PBS in a flat microplate. After being mixed for 2 min, HA was observed under a microscope. The minimum HA dose

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TABLE 1. Properties of PT and its subunits

PT or subunit	Sp act by[a]:					
	Hp-ELISA	SRBCM-ELISA	HA	CHO	LPF	IAP
PT	1.0	1.0	1.0	1.0	1.0	1.0
S1	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
S234	0.62	0.5	0.29	<0.01	<0.01	<0.01
S5	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

[a] Specific activities (per microgram of protein) of the subunits are expressed as relative values against the specific activities of PT (PT Ref 3). See the text for details.

of PT Ref 3 was 0.1 μg per well. The activity of HA inhibition of the antibody was estimated by assaying the HA activity of the mixture of PT (25 μl) and antibody (25 μl) diluted serially with PBS in the microplate as described above. Other HA procedures were essentially the same as reported previously (14).

CHO cell-clustering activity of PT was assayed by using CHO cell K1 as reported by Hewlett et al (7). The minimum clustering dose of PT Ref 3 was 5 pg/5 × 10^3 cells per well.

In vivo assay of PT activity. LP activity was measured by intravenous injection of the test samples and PT Ref 3 into five female SPF SLC-DDY, 4-week-old mice (Shizuoka Laboratory Animal Center, Hamamatsu, Japan) and was calculated in LP units from the relative value to PT Ref 3 (7,000 LP U/ml) as reported previously (13). Leukocytes (WBC) in tail vein blood 3 days after injection were counted. The WBC count in this paper shows the net count (ΔWBC) obtained by subtraction of the count of normal mice.

For measurement of IA activity, the mice were fasted overnight after WBC count for the LP assay. Blood (20 μl) taken from the tail vein of the five mice 15 min after intraperitoneal (i.p.) injection of 50% glucose (0.5 ml per mouse) was pooled in PBS (400 μl) for a fivefold dilution and was centrifuged to remove the blood cells. The insulin in the sera was measured by radioimmunoassay.

Isolation of subunits of PT. PT consists of five subunits, S1, S2, S3, S4, and S5, named in the order of their molecular size from largest to smallest, as revealed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (18, 20). The methods of dissociation of PT and isolation of the subunits were essentially the same as in the previous report (18). Purified PT was dissociated to subunits with 8 M urea, and each subunit was separated by successive Sephacryl S-200 gel filtration and CM- and DEAE-Sepharose CL-6B column chromatography. Since S1, S5, and S234 complex were obtained at high yield, these three subunits and the complex were employed routinely for the selection of hybridomas. For the characterization of a certain MAb, S4, S24, and S34 were also used, but S2 and S3 were not used because the yield of these subunits was rather poor. It was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis that the molecular size of the subunits isolated was not altered by the isolation procedures.

Assay of binding activity of the antibodies to PT and its subunits. Binding activity of the antibodies to the antigens was measured by the ELISA system, performed essentially by the method reported previously (13, 17). To assay the binding activity to PT combined with Hp (Hp-PT) or with SRBCM (SRBCM-PT), the microplates were coated overnight with (respectively) Hp (1 μg/ml) or SRBCM (20 μg/ml) dissolved in the coating buffer and then were overcoated with PT (0.25 μg/ml) dissolved in PBS containing 0.05% Tween 20. The antibody titer on ELISA was estimated from the dose-response curves by the parallel line assay method, using a reference antibody (anti-PT Ref 3). The anti-PT Ref 3 was pooled sera of mice hyperimmunized with pertussis toxoid (PTd) which was detoxified with Formalin and precipitated with aluminum hydroxide gel. The titer of the anti-PT Ref 3 was estimated as 450 ELISA U/ml on a PT-coated plate, using original reference antibody (anti-PT Ref 1) with a titer set arbitrarily as 2,700 ELISA U/ml (17). Binding activity of the antibodies was given by PT-ELISA, Hp-PT-ELISA, SRBCM-PT-ELISA, S1-ELISA, S234-ELISA, or S5-ELISA units according to the coating antigen.

Assay of neutralizing activity of antibodies against LP and IA activities of PT. Antibody preparations diluted serially were mixed with an equal volume of PT Ref 3 diluted with PBS containing 0.2% gelatin to a concentration of 0.8 μg/ml (28 Hp-ELISA U/ml). After reacting for at least 30 min at room temperature, 0.2 ml of the mixture was injected into the tail vein of mice. Five mice per dose were employed. At 3 and 4 days after injection of the mixture, leukocytes and blood insulin, respectively, were measured as described above, and the percentages of LP and IA activities neutralized were estimated, Neutralization units of anti-PT antibody could be calculated from the relative activity to anti-PT Ref 3 (450 U/ml) by the parallel line assay method.

Preparation and selection of hybridoma-producing specific antibody. A BALB/c mouse (Charles River Japan, Kanagawa, Japan) was primed three times with 17 μg of PTd emulsified with Freund incomplete adjuvant every 2 days by one i.p. and two subcutaneous injections and one more time with 20 μg of PT by subcutaneous injection 4 days after the third injection. Three months later, 25 μg of PTd was given intravenously. Three days after the booster injection, spleen cells were fused to SP2/O-Ag14 myeloma cells at a ratio of 10:1 by the methods described in the previous paper (11a). After selection of hybrid cells with hypoxanthine-aminopterin-thymidine medium (11), the presence of anti-PT antibodies in the culture fluids was tested by ELISA, using a microplate coated with 8 M urea-treated toxin in which the inner antigenic sites of the toxin might also be exposed. Of

TABLE 2. Binding activity of the three MAbs to PT or its subunits

Antibody	Binding activity (ELISA U/ml) to[a]:					
	PT	S1	S234	S5	Hp-PT	SRBCM-PT
1B7	2,000	6,100	<0.1	<0.1	2,340	2,700
3F10	1,580	3,960	<0.1	<0.1	1,170	1,760
1H2	410	<0.1	950	<0.1	126	41
Anti-PT Ref 3	450	450	450	450	450	450

[a] The binding activity of three MAbs to each antigen is expressed as relative values against the activity of anti-PT Ref 3 (450 ELISA U/ml) for each activity.

TABLE 3. Neutralization to the LP and IA activities of PT with the three MAbs

Antibody	Dose per mouse[a]	Neutralizing activity to:			
		LP		IA	
		%[b]	ED_{50}[c]	%[b]	ED_{50}[c]
1B7	10	85 ± 3	0.26	98 ± 5	0.23
	2	81 ± 2		89 ± 7	
	0.4	60 ± 2		54 ± 2	
	0.08	31 ± 2		35 ± 2	
3F10	10	41 ± 4	31.6	60 ± 7	3.8
	2	30 ± 7		43 ± 10	
	0.4	15 ± 5		27 ± 5	
	0.08	5 ± 2		5 ± 3	
1H2	10	28 ± 5	518	51 ± 7	9.2
	2	17 ± 4		33 ± 10	
	0.4	8 ± 4		20 ± 7	
1B7 + 3F10 (1:1)[d]	4	89 ± 5	0.1	97 ± 5	0.06
	0.8	89 ± 7		95 ± 8	
	0.16	54 ± 2		75 ± 10	
	0.032	31 ± 2		31 ± 3	
1B7 + 1H2 (1:1)	4	78 ± 5	0.32	78 ± 10	0.26
	0.8	77 ± 10		71 ± 8	
	0.16	37 ± 3		49 ± 10	
	0.032	16 ± 4		17 ± 6	
3F10 + 1H2 (1:1)	4	39 ± 7	24	43 ± 10	ND[e]
	0.8	19 ± 5		66 ± 11	
	0.16	10 ± 5		ND[e]	
1B7 + 3F10 + 1H2 (1:1:1)[d]	6	91 ± 4	0.15	98 ± 4	0.09
	1.2	91 ± 3		86 ± 5	
	0.24	63 ± 6		60 ± 6	
	0.048	29 ± 5		ND[e]	
Anti-PT Ref 3	9 U	95 ± 3	1.8 U	99 ± 2	0.8 U
	1.8 U	46 ± 3		74 ± 5	
	0.36 U	10 ± 4		28 ± 3	

[a] Doses of the three MAbs (1B7, 3F10, and 1H2) and of anti-PT antibody (anti-PT Ref 3) are expressed as micrograms of protein and PT-ELISA units, respectively. PT-ELISA units of the MAbs can be calculated by using the specific activity of each MAb; the specific activities of 1B7, 3F10, and 1H2 were 10.0, 4.0, and 0.55 PT-ELISA U per μg, respectively.

[b] Neutralizing activity is expressed as the percentage of the toxin activity neutralized. Each value is the average of four experiments ± standard error.

[c] ED_{50}, Dose neutralizing 50% of the test toxin (0.08 μg), expressed in micrograms of protein or PT-ELISA units (U).

[d] Each MAb was mixed with the same amount of protien.

[e] ND, Not determined.

180 growing hybrid cell cultures, 67 were antibody positive by the EILSA method. Antibodies in the 67 cultures were assayed for binding activity to the subunits by S1-ELISA, S234-ELISA, and S5-ELISA systems as well as to PT by PT-ELISA. Hybridoma-producing antibodies reacting with S1 were obtained at a high frequency. As a consequence of repeated cloning by limiting dilution and repeated checking of the binding activity in the ELISA systems, two hybridomas (1B7 and 3F10) producing MAbs to S1 and one (1H2) producing MAbs to S4 were selected for this study. The hybridoma and its MAb were given the same name. Specific binding of MAbs 1B7 and 3F10 to S1 and of MAb 1H2 to S4 was also confirmed by the immunoblotting method with sodium dodecyl sulfate-polyacrylamide gel electrophoresis of PT. The culture fluid of each hybridoma was concentrated and tested for the isotype of MAb by the double immunodifusion test, using anti-mouse immunoglobulins (Bionetics, Inc., Kensington, Md.). 1B7 was IgG2a, and both 3F10 and 1H2 were IgG1.

Preparation of MAb. To obtain a high titer of the antibodies, the hybridomas were injected into BALB/c mice primed with pristane (2,6,10,14-tetra-methyl pentadecane; Aldrich Chemical Co., Inc.). The antibodies in the ascites fluid were precipitated with ammonium sulfate at 50% saturation and then were purified by affinity chromatography with Protein A-Sepharose (Pharmacia Fine Chemicals) (4) or DEAE Affi-gel blue (Bio-Rad Laboratories) (2). All IgG preparations purified by either type of affinity chromatography showed one band in disc electrophoresis at pH 9.4 and showed the same specific activities in both binding and neutralizing activities to PT. The protein concentration of the purified IgG was estimated from the absorbance at 280 nm, using $E^{1\%} = 15$. All experiments except for the mouse protection test by i.c. challenge were performed with the purified IgG preparation of MAbs.

Assay of mouse-protecting activity of the antibodies. Two challenge systems, i.c. and aerosol challenge, were used in the passive protection test with the antibodies. In the i.c. challenge system, antibodies were injected i.p. into 4-week-old female SPF SLC-DDY mice. Three hours later, the mice were challenged i.c. with *B. pertussis* 18323 (5×10^4 cells per mouse). After observation for 2 weeks, the survival rate was determined.

In the aerosol challenge system, mice (7-day-old SPF SLC-ICR) were given i.p. 0.2 ml of antibodies diluted appropriately. Two hours later, aerosol infection in the mice was performed by the method reported previously (15). After inhalation, WBC in the tail vein were counted at appropriate times to check the specific process of pertussis infection. The WBC count 3 weeks after inhalation often reflected the severity of the disease and the grade of infection. When a mouse was dead under observation, the average WBC count (2×10^5 per μl) of dying mice was applied to the mouse as the number of WBC for calculation of the geometric mean of ΔWBC counts. Five weeks after infection, blood was taken from the survivors to titrate the antibodies against PT and FHA by ELISA and of agglutinin against formalinized *B. pertussis* 18323 as reported previously (16).

Statistical analyses. The 50% effective doses of the antibodies against LP or IA activity of PT were calculated from the regression lines of the toxin neutralization rate on the antibody dose. The 50% protective dose and its confidence interval were estimated by probit analysis.

RESULTS

Characterization of PT subunits. Table 1 shows some properties of the subunits used for evaluation of the MAbs isolated. Both S1 and S5 showed neither binding activity to Hp or SRBCM in the ELISA system nor HA activity, whereas S234 showed all three activities. These activities of S234 were also shown by S24 or S34. Although the specific activities of S24 were almost the same as those of S234, those of S34 were somewhat lower than those of the other two. The addition of S15 often enhanced the HA activity of S234. IA and CHO cell-clustering activities were not detected in any subunit, but these biological activities were fully restored when all of the subunits were mixed together.

Toxin-binding activity of MAbs. Table 2 shows the binding activity of the MAbs to PT, S1, S234, S5, and PT combining

TABLE 4. Protective activity of the three MAbs by the i.c. challenge system

Antibody injected			
P_{50}[a]	PT-ELISA U per mouse	Survival rate[b]	PD_{50}[c]
1B7	2,500	9/10	127 (50–326)
	500	16/20	
	100	8/20	
	20	4/20	
3F10	2,500	0/20	ND[d]
	500	0/20	
	100	0/20	
1H2	2,500	0/20	ND[d]
	500	0/20	
	100	0/20	
1B7 + 3F10 (1:1)[e]	1,000	17/20	173 (83–360)
	200	13/20	
	40	2/20	
1B7 + 1H2 (1:1)	1,000	7/10	465 (132–1,632)
	200	3/10	
	40	0/10	
1B7 + 3F10 + 1H2 (1:1:1)[e]	1,500	8/10	431 (134–1,390)
	300	4/10	
	60	1/10	
	12	0/10	
Anti-PT	250	6/10	212 (87–517)
	50	0/10	
	10	0/10	
PBS		0/20	

[a] MAbs and anti-PT antibody were precipitated with 50% saturation of ammonium sulfate (P_{50}) from ascites fluid containing MAb and from conventional anti-PT mouse serum.
[b] Number surviving/total mice.
[c] PD_{50} (50% protective dose, in PT-ELISA units per mouse) and its confidence interval ($P = 0.05$) in parentheses are indicated.
[d] ND, Not determined.
[e] Each MAb was mixed with the same number of PT-ELISA units.

Hp or SRBCM. The binding activity of each MAb to the counterpart subunit was higher than that to PT; both 1B7 and 3F10 gave the highest titer in S1-ELISA, whereas 1H2 did so in S234-ELISA. Furthermore, the binding activity of 1B7 and 3F10 in Hp-PT-ELISA or SRBCM-PT-ELISA was at almost the same level as that in PT-ELISA, but the activity of 1H2 in the former two was much lower than that in the latter ELISA system. This result, as well as those shown in Table 1, supported the idea that the S4 was related to the binding of PT to Hp or SRBCM.

Toxin-neutralizing activity of MAbs. Neutralizing activities of MAbs to LP and IA activities of PT were measured (Table 3). 1B7 was able to neutralize both biological activities, but 3F10 and 1H2 showed little neutralization of either activities. There was no significant difference between the 50% effective dose (in PT-ELISA units) of 1B7 (2.6 and 2.3 U for neutralization of LP and IA activities, respectively) and that of anti-PT Ref 3 (1.8 and 0.8 U for the two activities, respectively). When 1B7 was administered to the mice 1 day after intravenous injection of PT, leukocytosis was suppressed within 1 day (data not shown). HA inhibition of MAbs was also tested by using the HA inhibition system, with PT or S234 having HA activity. 1H2 inhibited HA with both PT and S234, but 1B7 and 3F10 inhibited only the HA with PT.

Mouse-protecting activity of MAbs by the i.c. challenge system. Table 4 shows the mouse protective activity of 50% saturated preparations of the three MAbs and their mixtures against i.c. challenge with *B. pertussis* 18323 cells. Only 1B7, which showed toxin neutralizing activity, could protect mice from the i.c. challenge. All mice administered the other MAbs, 3F10 or 1H2 or both, were dead within 1 week after challenge. The 50% protective dose based on PT-ELISA units of the 1B7 was almost the same as that of the conventional anti-PT antibody.

Mouse-protecting activity of MAbs by the aerosol challenge system. In the aerosol challenge system also, only 1B7 showed mouse-protective activity. 3F10 or 1H2 or both did not protect mice despite mild infection, as evaluated by ΔWBC count and survival rate of nonimmunized PBS control mice, and did not show any synergistic effect on 1B7

TABLE 5. Protective activity of the three MAbs against sublethal aerosol challenge

Antibody	Dose per mouse[a]	ΔWBC at 3 wk	Survival rate at 5 wk[b]
1B7	20	200	24/24
	4	1,000	22/24
	0.8	1,600	21/24
	0.16	10,000	15/24
	0.032	30,000	7/24
3F10	20	45,000	7/24
	4	30,000	6/24
	0.8	30,000	7/24
1H2	20	34,000	10/24
	4	32,000	8/24
	0.8	35,000	9/24
1B7 + 3F10 (1:1)[c]	8	200	7/8
	0.8	4,600	6/8
	0.08	21,000	4/8
1B7 + 1H2 (1:1)	8	4,000	7/8
	0.8	10,400	5/8
	0.08	19,900	4/8
3F10 + 1H2 (1:1)	8	51,000	3/8
	0.8	55,000	3/8
	0.08	33,500	2/8
1B7 + 3F10 + 1H2 (1:1:1)[c]	2.4	4,500	24/24
	0.48	13,000	18/24
	0.096	20,000	14/24
Anti-PT Ref 3	18 U	5,000	15/16
	3.6 U	18,000	8/16
	0.72 U	20,000	7/16
	0.36 U	43,000	5/16
PBS		44,500	5/16

[a] Doses of the three MAbs (1B7, 3F10, and 1H2) and anti-PT antibody (anti-PT Ref 3) are expressed as micrograms of protein and PT-ELISA units (U), respectively. PT-ELISA units of the MAbs can be calculated by using the specific activity of each MAb; the specific activities of 1B7, 3F10, and 1H2 were 10.0, 4.0, and 0.55 PT-ELISA U per μg, respectively.
[b] Number surviving/total mice.
[c] Each MAb was mixed with the same amount of protein.

TABLE 6. Protective activity of the MAb 1B7 against lethal aerosol challenge

Antibody	Dose per mouse[a]	Survival rate at day[b]:			PD_{50}[c]	Antibody titer[d] of survivor to:		
		9	20	35		PT	FHA	Agg[e]
1B7	20	8/8	8/8	8/8	1.5 (0.6–3.9)	45 (16–128)	230 (141–395)	57 (14–226)
	4	8/8	8/8	7/8		56 (21–150)	205 (132–318)	49 (9–274)
	0.8	8/8	5/8	2/8		28 (2–256)	227 (2–26,000)	10 (10–10)
	0.16	8/8	1/8	0/8		ND[f]	ND	ND
	20[g]	6/6	5/6	3/6		47 (6–357)	91 (2–4,860)	202 (75–545)
Anti-PT[h]	100 U	8/8	7/8	7/8	35 U (14–85)	29 (2–452)	118 (34–359)	238 (63–896)
	30 U	8/8	7/8	3/8		51 (2–1,320)	126 (3–5,000)	80 (1–7,800)
	10 U	8/8	2/8	1/8		1	9	40
	3 U	8/8	1/8	0/8		ND	ND	ND
	100 U[g]	6/6	3/6	3/6		21 (1–359)	231 (144–370)	80 (1–3,900)
PBS		8/8	0/8	0/8		ND	ND	ND

[a] Doses of 1B7 and anti-PT antibodies are expressed as micrograms of protein and PT-ELISA units (U), respectively. See footnote *a* of Table 3.
[b] Number surviving/total mice on the day after aerosol challenge.
[c] The 50% protective doses (PD_{50}s) of 1B7 and anti-PT antibodies are expressed as micrograms of protein and PT-ELISA units (U), respectively. The 95% confidence intervals are indicated in parentheses.
[d] Geometric mean of ELISA units or agglutinin titers and the confidence interval ($P = 0.05$) in parentheses are indicated.
[e] Agg, Agglutinogens of *B. pertussis* 18323.
[f] ND, Not determined because no mice survived.
[g] These antibodies were administered on day 9 after challenge.
[h] The same antibody preparation to anti-PT (P_{50}) as in Table 4.

(Table 5). The results are in accord with the results of neutralizing activity of MAbs to LP and IA activity in Table 3.

The protective effect of 1B7 on mice subjected to a lethal aerosol challenge was examined more precisely (Table 6). The time course of leukocytosis in the mice (Fig. 1) and the survival rate at days 9, 20, and 35 after the challenge (Table 6) showed that 1B7 was as potent a protective antibody as polyclonal conventional anti-PT antibody; the 50% protective doses values of 1B7 and the conventional antibody were 1.5 μg (15 PT-ELISA U) and 35 PT-ELISA U per mouse, respectively. All of the mice immunized with doses of 20 μg of 3F10 or 1H2 or both were dead within 3 weeks after aerosol, as in the case of the nonimmunized PBS control mice (data not shown). Even on day 9, when severe leukocytosis had already appeared, 50% of the mice were cured by the administration of 1B7 as well as the conventional antibody; otherwise the mice died by day 9 (Fig. 1 and Table 6). All surviving mice acquired antibodies against the antigens derived from the aerosolized organism.

DISCUSSION

MAb 1B7 showed high LP or IA neutralization and mouse protection activities, but 3F10 did not show either, although both antibodies had the same specificity for subunit S1, which had ADP-ribosylating activity to putative membrane protein Ni (3, 8) but had no binding activity for Hp or SRBCM. The antibody 1H2 with specificity for S4, which played an important role in the binding of the PT molecule to Hp or SRBCM, was expected to show a toxin neutralization, as in the case of MAbs to diphtheria (5, 23) and cholera toxins (9; Y. Imagawa, T. Maeda, E. Tokunaga, K. Kudo, T. Muraoka, and S. Otomo, Jpn. J. Med. Sci. Biol., in press). However, it inhibited neither LP nor IA activity of PT. Of course, this does not mean that every antibody to S4 could not prevent toxin action in vivo. Because anti-S234 conventional antibodies without any contamination of anti-S1 antibody neutralized LP activity (18; unpublished data), it may be possible that some MAbs to S2, S3, or S4 show the neutralizing activity to PT. We are now trying to obtain such MAbs. Although anti-ADP-ribosylating activity of the MAbs was not assayed in this study, we are preparing an assay of the anti-enzymatic activity for an understanding of the mode of action of PT and its antibody. The level of inhibitory activity of MAb on PT depends on which activity is measured. When the neutralizing activity of the MAbs to CHO cell-clustering activity of PT was examined, 1B7 and 3F10 but not 1H2 neutralized the activity. This neutralizing activity of each of the former two MAbs was lower than that of conventional anti-PT antibody, but that of the mixture of the two MAbs was almost equivalent to that of the conventional antibody (unpublished data). Furthermore, when the binding activity of the MAbs to PTd was examined by the ELISA method, the PT-ELISA/PTd-ELISA ratio of 1B7 was much higher than that of 3F10 or 1H2 (unpublished data). This ratio also depends on the PTd preparations used for the assay. Biological activity remaining in PTd detoxified with Formalin might be different according to the toxoiding conditions. Decreases in the activities of PT during toxoiding were not always parallel among Hp-ELISA, CHO cell-clustering, and LP activities; they were parallel under certain conditions (13) but not under other conditions of toxoiding. What is pertussis toxoid? This may be a new question.

The foregoing results indicated what diverse biological activities PT has and how important the selection of items that should be tested for toxicity in the pertussis vaccine is.

Focusing on the mouse-protecting activity of 1B7 against pertussis infection, its potency was almost the same as or superior to that of polyclonal conventional antibody (Tables 4 to 6). Neutralization of LP activity must be important for protection against pertussis infection or for therapy of the disease; even after severe leukocytosis occurred in the infections, the antibody 1B7 could suppress the leukocytosis and heal the disease as well as the polyclonal antibody did (Tables 3 and 5; Fig. 1). The synergistic effect of anti-FHA antibody on the action of 1B7 was observed when anti-FHA was given before infection but not after onset of the clinical signs (unpublished data). The titers of anti-PT, anti-FHA, and agglutinin antibodies produced by mice that resisted the disease and survived with 1B7 or anti-PT antibody (Table 6)

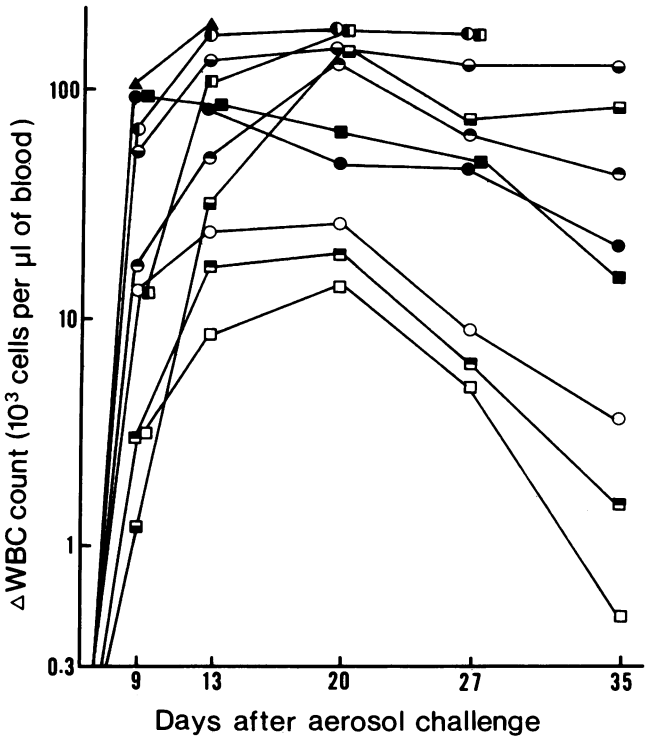

FIG. 1. Time course of WBC counts after aerosol infection of mice immunized with MAb 1B7 or conventional anti-PT. Data points of WBC counts are geometric means of the mice on the corresponding days. Symbols: □, ⬒, ⬓, and ◧, mice injected with MAb 1B7 at 20 (200), 4 (40), 0.8 (8.0), and 0.16 (1.6) μg (PT-ELISA U) per mouse, respectively; ■, mice injected with MAb 1B7 at 20 (200) μg (PT-ELISA U) per mouse on day 9 after aerosol infection; ○, ◓, ◒, and ◐, mice injected with anti-PT at 100, 30, 10, and 3 PT-ELISA U per mouse, respectively; ●, mice injected with anti-PT at 100 PT-ELISA U per mouse on day 9 after aerosol infection; ▲, nonimmunized (PBS) challenge control.

suggested that the mice permitted the organisms to grow in a restricted region at the initial stage of the infection. This was not the case with anti-FHA antibody (12). The foregoing results showed that MAb to PT might be as potent as the polyclonal conventional antibody in protecting mice against pertussis infection by either the i.c. challenge or the aerosol challenge system.

Application of the MAbs as the reference antibody for the assay of antigen or its antibody should be considered because we could take advantage of mass production of uniform antibody. Actually, the mixture of a few kinds of MAbs could be available as a reference instead of using the polyclonal conventional antibody. In this paper, we had to use both micrograms and PT-ELISA units to express the dose of antibody despite the complexity involved, because the ELSIA units we used were not common units decided by the use of international reference antibody or reference toxin. Such reference preparations are still not available, but now it might be not so difficult to prepare an international reference preparation by a collaborative study among several laboratories.

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